AD_____

AWARD NUMBER DAMD17-97-1-7252

TITLE: Retinoids and Retinoid Metabolism in Breast Cancer

PRINCIPAL INVESTIGATOR: Jisun Paik

CONTRACTING ORGANIZATION: University of Washington
Seattle, Washington 98195

REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Annual (16 Jun 97 - 15 Jun 98)

4. TITLE AND SUBTITLE
Retinoids and Retinoid Metabolism in Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7252

6. AUTHOR(S)
Jisun Paik

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Washington
Seattle, Washington 98195

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

19981218 065

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

My studies aim to understand vitamin A metabolism in breast tissue, specifically the role of the human 9 *cis*-retinol dehydrogenase (9cRDH). The 9cRDH gene was cloned from a normalized mammary library by Blaner and colleagues. We studied its expression in two normal human mammary epithelial cell strains (HMECs) and a panel of breast cancer cell lines. mRNA levels were determined by a semi-quantitative, multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) strategy. 9cRDH mRNA levels were similar among the cells tested, with the exception of the breast cancer cell lines, Hs578T and T47D, which exhibited higher expression than the normal strains, and MCF-7, which showed the lowest levels. Individual 9cRDH activity levels in normal and tumor cells, as measured by normal phase HPLC, paralleled expression levels observed by RT-PCR. In order to study the biochemical characteristics of 9cRDH and to determine optimal activity test conditions, we developed a Hep G2 cell line that expresses high levels of 9cRDH protein (L*RDH*SN/Hep G2). The 9cRDH cDNA was transduced into the cells using the LXSN retroviral vector containing the full length 9cRDH gene. The activity of 9cRDH tested in microsomes of L*RDH*SN/Hep G2 cells was time- and protein concentration-dependent and followed Michaelis-Menten kinetics.

14. SUBJECT TERMS
Breast Cancer , Retinoids, Metabolism, Therapy, Nutrition, Chemoprevention

15. NUMBER OF PAGES
22

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

JP
____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

JP
____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

JP
____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

JP ____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Paul Jones 7/14/98

PI - Signature Date

TABLE OF CONTENTS

Abstract

My studies aim to understand vitamin A metabolism in breast tissue, specifically the role of the human 9 *cis*-retinol dehydrogenase (9cRDH). The 9cRDH gene was cloned from a normalized mammary library by Blaner and colleagues. We studied its expression in two normal human mammary epithelial cell strains (HMECs) and a panel of breast cancer cell lines. mRNA levels were determined by a semi-quantitative, multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) strategy. 9cRDH mRNA levels were similar among the cells tested, with the exception of the breast cancer cell lines, Hs578T and T47D, which exhibited higher expression than the normal strains, and MCF-7, which showed the lowest levels. Individual 9cRDH activity levels in normal and tumor cells, as measured by normal phase HPLC, paralleled expression levels observed by RT-PCR. In order to study the biochemical characteristics of 9cRDH and to determine optimal activity test conditions, we developed a Hep G2 cell line that expresses high levels of 9cRDH protein (L*RDH*SN/Hep G2). The 9cRDH cDNA was transduced into the cells using the LXSN retroviral vector containing the full length 9cRDH gene. The activity of 9cRDH tested in microsomes of L*RDH*SN/Hep G2 cells was time- and protein concentration-dependent and followed Michaelis-Menten kinetics.

Introduction

Retinoids may negatively modulate the initiation , growth, and progression of breast cancer in cell culture and animal models (1-15). One mechanism by which retinoic acids affect cell growth is through binding and activation of the nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Compared to the explosive development in understanding the action of RARs and RXRs at the molecular level, little is known about how their ligands, all-*trans* and 9-*cis*-retinoic acids, are formed and how levels are regulated in tissues, including the mammary gland. It is generally accepted that all-*trans*-retinoic acid (at-RA) is formed by a consecutive oxidation process of all-*trans* retinol (at-ROL) by retinol dehydrogenase and retinaldehyde dehydrogenase, similar to alcohol metabolism (16). Indeed, two families of alcohol dehydrogenases, classes I and IV, have been investigated for their possible involvement in retinol metabolism (17-20). During the past 10 years, many retinol-specific enzymes (21-23) have been identified. These latter enzymes belong to the short chain dehydrogenase/reductase (SDR) family which metabolizes a diverse group of secondary alcohols and ketones, including steroids (estrogen, androgen, glucocorticoids) and prostaglandins (E_2, $F_{2\alpha}$) in human (24, 25). To date, close to 100 members have been reported from various species, including bacteria, plants and animals (26), and the list is still growing. Strict conservation of amino acid sequences among these family members is not very high (15-30%), which may explain their diverse substrates. On the contrary, the tertiary structures among some of the members show striking similarities despite their low sequence identity of 15% (25). Both retinol and alcohol dehydrogenases may participate in the oxidation of at-ROL to all-*trans*-retinaldehyde (at-RAL), the rate limiting step (16) in at-RA synthesis, likely in a tissue and developmental-specific pattern.

Since 9c-RA, one of the isoforms of at-RA, has been suggested as a biological ligand for RXRs (27, 28), efforts have been made to find the pathway for 9c-RA synthesis. The easiest explanation comes from the observation of the isomerization of at-RA to 9c-RA in cells and tissues (27-29). However, the possible involvement of isomerase(s) has not been proven unequivocally. Since then, several investigators have cloned the genes for *cis*-retinol dehydrogenases, those enzymes capable of metabolizing *cis*-retinols to their aldehydes (26, 30-35). The identification of these enzymes argues for the existence of yet another pathway for 9c-RA synthesis *in vivo*, in which 9c-ROL is converted to 9c-RA by two step oxidation as seen for at-ROL metabolism. The source of 9c-ROL is unknown,

though possible mechanisms are suggested, including isomerization from at-ROL (36) and cleavage from 9c-ß-carotene, though the latter may not be very efficient (37, 38). Also, 9c-ROL has been detected in kidney and liver in various species (39-41), indicating the existence of substrate for 9cRDH.

Blaner and colleagues isolated a cDNA from a normalized human mammary library and demonstrated that the gene product catalyzes 9c-ROL oxidation to 9c-RAL (35). They showed by Northern blot that the mRNA of this gene, 9cRDH, is abundant in normal mammary tissue but not in cancerous breast tissue. Our preliminary expression study using RT-PCR with various breast cancer cells and normal HMECs is in agreement with their data. Cancer cells often had diminished expression or even absence of expression while both HMECs tested seemed to have higher expression of the gene. However, an accurate quantitation couldn't be made. Thus, I developed a multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) to study the semiquantitation of 9cRDH in breast cells. I also created a human hepatoma cell line, L*RDH*SN/Hep G2, which overexpresses 9cRDH, to study biochemical characteristics of the enzyme in detail. The Hep G2 cell line was chosen because the liver is the main site for vitamin A metabolism which makes a liver cell a good model system. Furthermore, this line is known to produce various proteins, which are important for retinoid metabolism, including albumin and retinol binding protein (RBP) (42, 43).

My studies are designed to understand the precise pathways by which dietary or therapeutic retinoids are metabolized in the mammary gland.

Material and Methods

h9cRDH expression studies

RNAs from two normal HMECs and a panel of breast cancer cells were purified by guanidine isothiocyanate/cesium chloride centrifugation (44). Poly A^+ RNA was extracted using a mRNA isolation kit (Boehringer-Mannheim) according to the manufacturer's instructions. Reverse transcription was performed with 300 ng mRNA from each cell line or cell strain using Superscript RT®(Gibco) and random hexamers (Boehringer-Mannheim) at 42°C for an hour. The RT reaction (1µl) was used for subsequent PCR. Semiquantitative PCR strategy was applied to measure relative mRNA level in tested cells, adapted from Henegariu et al. (45). Primers for 9cRDH and for hypoxanthine-phosphoribosyltransferase (HPRT) were used in a same reaction tube to amplify both genes simultaneously. The HPRT primers were 1676-1696 (reverse:R) and 41481-41502 (forward:F) relative to its genomic sequence (46), resulting in a 705 base pair (bp)-product. The 9cRDH primers were 397-416 (F) and 851-870 (R) of the cDNA sequence resulting in a 473 bp-product. PCR conditions were in a total reaction volume of 25 µl with 3 pmole of each 9cRDH primer, 0.5 pmole of each HPRT primer, 3 mM $MgCl_2$, 1.5 U Taq-polymerase (Boehringer-Mannheim), 40 mM tetramethylammonium chloride (Sigma). Thermal cycling parameters were 94°C for 5 min, (94°C for 30 sec, 56°C for 30 sec, 68°C for 30 sec) x 40 cycles, and 68°C for 5 min. Resulting amplicons (15 µl) were analyzed on ethidium bromide stained-agarose gel. The intensity of signals were analyzed by densitometry (NIH image, http://sunny.dcrt.nih.gov/itc/gel/)

Transduction of Hep G2 cells with h9cRDH

Production of Hep G2 cells with h9cRDH:

Retroviral vector, pL*X*SN (a gift from Dr. A. Dusty Miller) was used to create cells which can stably produce retroviruses containing full length 9cRDH (47). The full length

9cRDH cDNA was directionally cloned into pL*X*SN using EcoRI and XhoI from pcDNA3 where it was originally cloned (35). Structures of vectors are shown in **Fig. 1**.

PE501, a packaging cell line, was transiently transfected with either empty vector (pL*X*SN) or vector containing 9cRDH (pL*RDH*SN) using calcium-phosphate precipitation (47). The viruses produced by PE501 were then used to infect another packaging cell line, PA317. This method was chosen since the resulting viruses are relatively homogeneous due to their origin from a single integrated virus while the viruses produced from direct transfection could come from multiple copies of various arrangement (47). The order of packaging cell lines was determined so that the virus would be produced from amphotropic cell line, PA317, which can infect subsequent human cells. Transduced cells were selected via G418 (0.75 mg/ml), and ten clones each were picked from cells containing either L*X*SN or L*RDH*SN. Clones were tested for virus titer, viral structural integrity, mRNA expression by vector titer test, Southern blot, and Northern blot, respectively (pages 7-8). A clonal cell line producing viruses with intact structure and high titer was later used to infect Hep G2 cells, a human hepatoma cell line. The resulting cell lines (mass culture) are referred as L*X*SN/Hep G2 and L*RDH*SN/Hep G 2. These two cell lines were utilized for kinetic studies on pages 8-9.

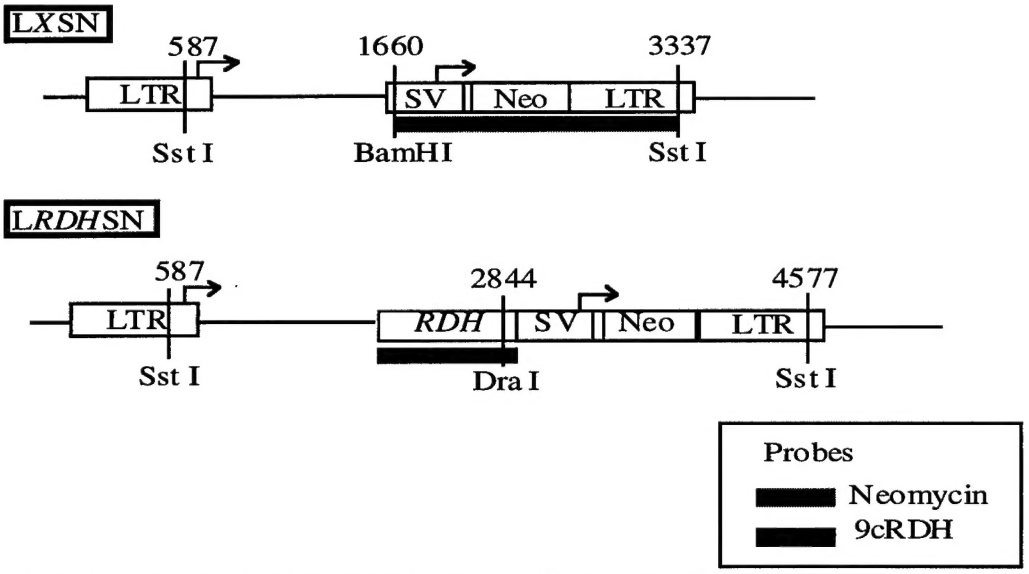

Figure 1. Structure of retroviral vectors. Structures of empty vector (L*X*SN) and vector containing 9cRDH (L*RDH*SN) are shown. Restriction enzyme sites that are used for Southern blot probes are also depicted. Sequences of the probes for Southern and Northern blots are drawn with dark bars.

Virus titer test:

NIH 3T3 cells were seeded at $5x10^5$ cells/ p60. The following day, cells were infected with 10, 100, and 1000 µl of virus containing media from PA317 clonal cells . Infected cells were then plated at 1:20 dilution and were cultured under G418 selection (0.75 mg/ml). When the mock transduced cells died off, colonies of transduced cells were stained with crystal violet and counted.

Southern blot:

Southern blot analysis was used to test integrity of the viral structure and to determine the copy number of the incorporated gene. Three clones each from PA317 cells transduced with either L*X*SN or L*RDH*SN were grown to confluency on p100 tissue culture plates and incubated at 37°C overnight in 10 mM Tris buffer containing proteinase K

(100 μg/ml), RNAse A (20 μg/ml), 0.5% SDS and 0.1 M EDTA. DNA was then extracted with phenol/chloroform. DNA (30 μg) from each clone was digested with either SstI or DraI overnight at 37°C and ran on 1% agarose gel in TAE buffer, followed by transfer to a nylon membrane (Zeta probe blotting membrane, BioRad). ^{32}P labeled probes (**Fig. 1**) were prepared from either neomycin resistance gene or from full length 9cRDH using a Random prime labeling kit® (Boehringer-Mannheim).

Northern blot:

RNA was extracted by guanidine isothiocyanate/cesium chloride centrifugation as described above from the same PA317 clonal cell lines as ones used for Southern blot analysis. Total RNA (20μg) was loaded onto 1.2% denaturing agarose gel and blotted onto a nylon membrane (Zeta probe blotting membrane, BioRad). The membrane was subsequently hybridized with the probes described above.

9cRDH activity test

Retinoids:

All-*trans*-, 13-*cis*-, 9-*cis*-retinal, all-*trans*, and 13-*cis*-retinol were purchased from Sigma. The internal standard, all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol (TMMP-ROH=Ro12-0586) (48) was obtained from Dr. Louise H. Foley of Hoffmann-LaRoche, Inc. (Nutley, NJ). 9c-ROL was prepared by reducing 9c-RAL (Sigma) with $NaBH_4$ (Sigma) followed by purification by normal phase HPLC (35).

Enzyme kinetic studies:

Microsomes of L*RDH*SN/Hep G2 cells were prepared by the method of Fleischer and Kervina (49) with some modifications. Briefly, cells were grown on p150 to confluency, washed twice with ice cold PBS, and collected by scraping into a buffer containing 25 mM Tris, pH 7.4, 0.25 M sucrose, and 1 mM DTT. Cells were homogenized with a Dounce homogenizer (B) with 50 strokes on ice and centrifuged at 12,000 g for 10 minutes at 4°C. The resulting pellet contains mitochondrial and nuclear proteins and is referred to as the heavy particle fraction. Supernatants were centrifuged at 100,000 g for 1 hour at 4°C, and the microsome pellets were resuspended in the same buffer without sucrose. Aliquots were quick frozen in ethanol-dry ice bath and kept at -70°C until use. Protein concentration was measured using Bradford assay (BioRad) per manufacturer's recommendation.

All the following procedures for enzyme activity test were performed under dim red lights to minimize photoisomerization and were a modification of the method by Mertz et al. (35). In total reaction volume of 600 μl, 30 μg of microsomes were incubated at 37°C for 20 min with 9c-ROL (in ethanol) at various concentrations (0, 1, 2.5, 5 and 10 μM), 2 mM NAD^+ in a buffer containing 10 mM Hepes, pH 8.0, 150 mM KCl, and 1 mM EDTA. The total volume of ethanol in the reaction did not exceed more than 10% of total reaction volume. Reaction tubes were flushed with nitrogen before sealing with parafilm and covered with aluminum foil to minimize contact with O_2 and light. Following the incubation, proteins were denatured with an equal volume of 100% ethanol, and the internal standard, TMMP-ROH, was added to each reaction. Retinoids were extracted in 2.5 ml hexane and the extracts were backwashed with 0.5 ml deionized water and dried under gentle nitrogen stream. Samples were reconstituted in 130 μl of mobile phase (hexane: ethyl acetate: butanol) and analyzed by normal phase HPLC (see below).

HPLC analysis:

Samples were separated on a 4.6 x 15 cm LC-SI Supelcosil® column (Supelco, Belefonte, PA) preceded by a silica guard column (Supelco) using hexane: ethyl acetate:

butanol (96.9:3:0.1, v/v) as mobile phase with flow rate at 0.8 ml/min. Isomers of retinols and retinaldehydes were detected at absorbances of 325 and 365 nm, respectively, using a Waters 996 photodiode array detector. Peaks were identified by comparing their retention times and spectra to those of pure retinoid standards. Each isomer of retinols and retinaldehydes was quantitated by calculating the integrated area under the peak against that of known standards. The loss during extraction was accounted for by adjusting the values of retinoids to percent recovery of the internal standard.

Enzyme activity test in Hep G2 cell and breast cells

Cell culture:

Modified Eagle's medium (α-MEM) and bovine pituitary extract (BPE) were purchased from Gibco; insulin, epidermal growth factor (EGF), hydrocortisone, and dimethyl sulfoxide (DMSO) were purchased from Sigma. Mammary epithelial cell basal medium (MEBM) was purchased from Clonetics. Fetal bovine serum (FBS) was purchased from Hyclone. G418 Sulfate was purchased from Calbiochem.

Cells were grown in monolayers. Breast cancer cells were grown in the α-MEM supplemented with 5% fetal bovine serum (FBS), insulin (1 μg/ml), EGF (12.5 ng/ml), and hydrocortisone (1 μg/ml). Normal HMECs were grown in MEBM supplemented with 0.004% BPE, insulin (1 μg/ml), EGF(10 μg/ml), hydrocortisone(0.5 μg/ml), and isoproterenol (0.01 mM). Transduced Hep G2 cells were grown in the α-MEM supplemented with 10% FBS and G418 (0.75 mg/ml).

Enzyme activity test:

At confluency, cells were washed twice with ice-cold PBS, collected by scraping, and stored at -80°C in freezing media (α-MEM with 10% DMSO and 20% FBS). Upon use, cells were thawed, washed twice with ice cold PBS, and centrifuged to pellets. Cell pellets were then resuspended in buffer containing 10 mM Hepes, pH 8.0, 150 mM KCl and 1 mM EDTA and homogenized in a dounce homogenizer on ice. Determination of protein concentration and of enzyme activity were performed as described in kinetic studies (page 8) except that 100 μg protein of whole cell homogenates and 10 μM 9c-ROL were used, with an incubation time of 60 minutes.

Immunoblot analysis

L*X*SN/Hep G2 and L*RDH*SN/Hep G2 cells were prepared for microsomes as described in page 8. Microsome- and cytosolic proteins (20-50 μg each) were loaded onto 15% SDS-PAGE gels and transferred on to polyvinylidene difluoride (PVDF) membrane at 30V overnight at 4°C. 9cRDH protein was detected by incubating the membrane with anti-peptide (amino acid sequence 236-257) polyclonal antibody raised in a rabbit (W. Blaner's laboratory) and anti-rabbit secondary antibody (Santa Cruz) followed by ECL detection (Pierce).

Results

mRNA expression of breast cells

Multiplex PCR enabled us to semiquantitatively assess the level of 9cRDH mRNA in two HMECs and a panel of breast cancer cell lines. Relative intensity of signals from HPRT and 9cRDH of each cell was calculated by densitometry (**Fig. 2**).

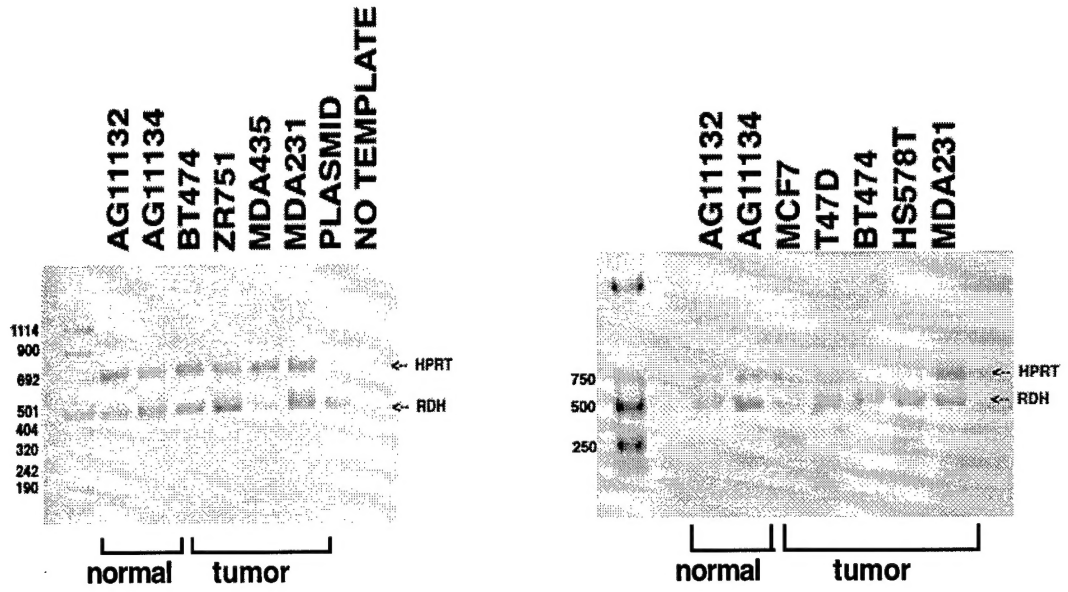

Figure 2. An example of multiplex RT-PCR. mRNA (300 ng) of each cell was used for reverse transcription (RT). RT reaction (1 μl) was used for the following multiplex PCR. The HPRT primers were 1676-1696 (R) and 41481-41502 (F) relative to its genomic sequence and the 9cRDH primers were 397-416 (F) and 851-870 (R) of the cDNA sequence. PCR conditions were in total 25 μl reaction with 3 pmole of each 9cRDH primer, 0.5 pmole of each HPRT primer, 3 mM $MgCl_2$, 1.5 U Taq-polymerase, 40 mM tetramethylammonium chloride. Thermal cycling parameters were 94°C for 5 min, (94°C for 30 sec, 56°C for 30 sec, 68°C for 30 sec) x 40 cycles, and 68°C for 5 min. Amplicons (15 μl each) were analyzed on ethidium bromide stained-agarose gel. The intensity of signals was analyzed by densitometry.

The calculated ratio of 9cRDH to HPRT from each cell was expressed as a relative term to that of AG11132. Experiments were repeated 4-5 times and the values were averaged. mRNA levels varied 5-fold among tested cells, with MCF-7 being the lowest and Hs578T and T47D being the highest (**Fig. 3**).

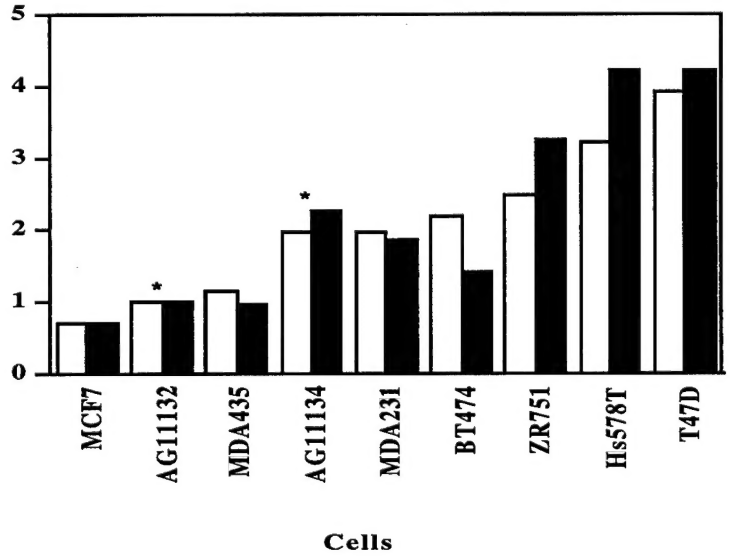

Figure 3. Summary of expression- and activity-level of 9cRDH in breast cells. mRNA expression of 9cRDH was measured by multiplex PCR, and the enzyme activity level was measured by normal phase HPLC. mRNA and activity levels of AG11132 were arbitrarily set as 1. White bars represent relative expression levels and black bars represent activity levels of 9cRDH in each cell line or strain. Normal HMECs are marked with an *.

Transduction of Hep G2 cells with h9cRDH

Since the enzyme was not expressed at high enough levels in any of breast cells to further characterize the biochemical properties of this enzyme, we needed to overexpress the protein. For this purpose, human hepatoma cell line, Hep G2 was chosen since the liver is known to play a major role in vitamin A metabolism, and Hep G2 cells were reported to produce diverse proteins such as albumin and RBP (42, 43). A retroviral vector was used to overexpress the enzyme since unlimited amount of viral particles can be obtained from a packaging cell line, which can then be used for subsequent gene transduction into any proliferating, cultured human cell.

Among ten clones of PA317 cells, three clones were tested by Southern blot for the structural integrity of the viral vector and the copy number of incorporated gene (**Fig. 4**). DNA (30μg) was digested with either Sst I or Dra I, which cut either side of LTR and h9cRDH, respectively (**Fig. 1**). If the viral structure is intact, SstI digest will produce 2750bp- (L*X*SN) and 3990 bp-signal (L*RDH*SN) when probed with the Neomycin gene. With Dra I digestion and a 9cRDH probe, a 2844+ bp-signal should be visible from L*RDH*SN transduced cells. All three clones of L*X*SN transduced cells showed single band of expected size when probed with Neomycin and no signal was seen when probed with h9cRDH as expected. However, only one clone of L*RDH*SN transduced cells showed a single band of the expected size with SstI digestion when probed with Neomycin. This was confirmed by hybridizing the same blot with 9cRDH where the endogenous sequence was also detected.

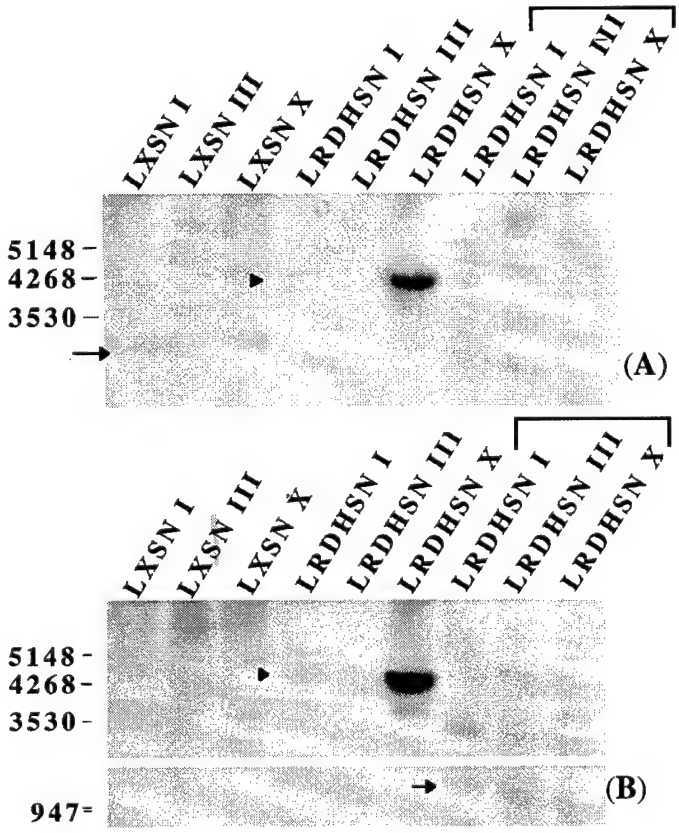

Figure 4. Southern blot analysis. DNA (30 μg) of three clones each were digested with either SstI or DraI (marked with bracket) and probed with either (**A**) Neomycin or (**B**) 9cRDH. All three L*X*SN transduced cells showed expected ~2750 bp-signals (arrow) when probed with neomycin but no signal was observed when probed with 9cRDH. Only one clone (L*RDH*SN I) of 9cRDH transduced cells show expected size band of 3990 bps (arrow head) when probed with either neomycin or 9cRDH. When the same DNA was digested with DraI (bracket), three different size bands were seen in each clones due to the gene incorporation at different sites. Endogenous gene can also be seen in all three clones of L*RDH*SN/Hep G2, which show three identical size bands (arrow) in (**B**)

RNA expression of transduced genes in the same clonal cells tested by Southern blot analysis, was analyzed by Northern blots probed with either Neomycin or the full length 9cRDH probe (**Fig. 5**). All three L*X*SN transduced cell lines had two transcripts driven by either LTR or SV-40 viral promotors when probed with Neomycin, but no signal was detected when 9cRDH was used as a probe. On the other hand, again only one clone of L*RDH*SN showed the two transcripts of expected sizes resulting from two possible poly A sites in the viral construct (one in inserted 9cRDH cDNA and the other in LTR).

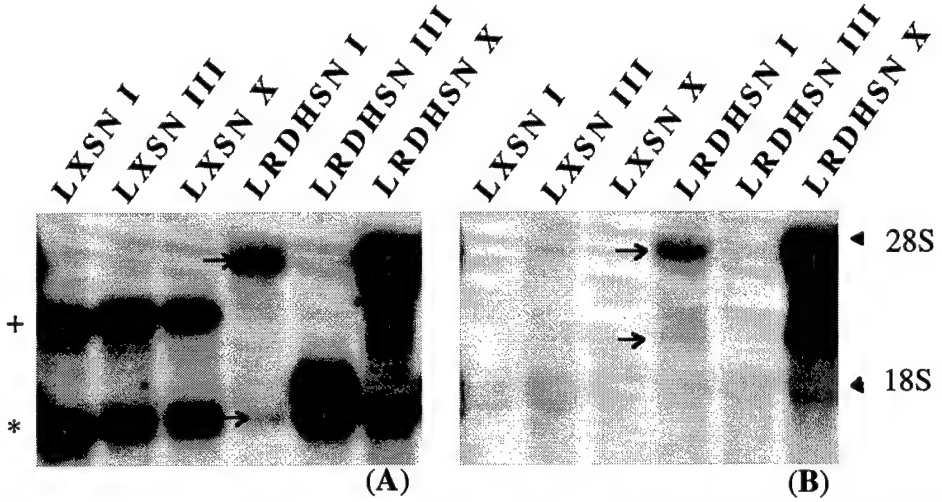

Figure 5. Northern blot analysis of three clonal cell lines. Total RNA (20 µg) from each cell line was analyzed with (**A**) Neomycin and (**B**) 9cRDH probe. The two expected transcripts driven by either SV (*) or LTR promotor (+) were detected from L*X*SN transduced cell lines with Neomycin probe (~ 1.5 kb and ~ 3^{+} kb) . However, no signal was detected from the same cell lines when 9cRDH was used as a probe. Only one clone, L*RDH*SN I, has the expected size-signal when tested with either neomycin probe or 9cRDH probe (arrow).

Virus titers of L*X*SN clones were between 120,000 and 350,000 CFU/ml and that of the intact L*RDH*SN clone was 20,000 CFU/ml. Viruses with intact structure were later used to transduce Hep G2 cells. Both L*X*SN- and L*RDH*SN/ Hep G2 cells (mass culture) were examined by northern blot. The same results were observed as seen in PA317 cells transduced with 9cRDH (**Fig. 6**).

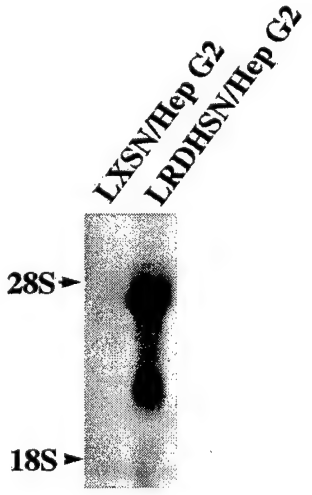

Figure 6. Northern blot analysis of transduced Hep G2 cells. Full length 9cRDH probe was prepared using Random prime kit. Two transcripts were observed from L*RDH*SN/Hep G2 cells, which results from two possible Poly A sites in the vector construct. No signal was visible from L*X*SN/Hep G2 cells, as expected. Note: Endogenous levels of 9cRDH mRNA can only be detected by RT-PCR.

To confirm the expression of protein, an immunoblotting assay with antipeptide antibody was performed, and a protein of the expected size of ~ 32 kD was detected in microsomal fraction of L*RDH*SN/Hep G2 cells. (**Fig.7**).

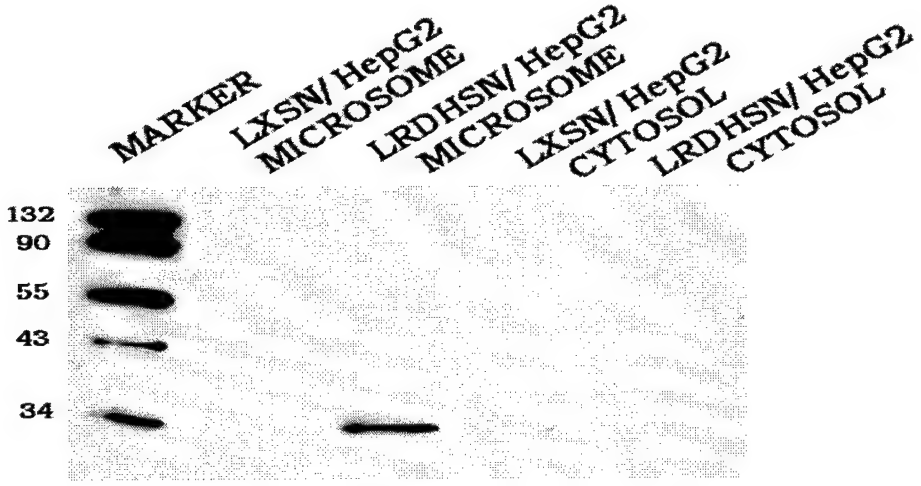

Figure 7. Immunoblot analysis of 9cRDH in transduced Hep G2 cells. Subcellular fractions of L*X*SN- and L*RDH*SN/Hep G2 cells were tested to examine the existence of 9cRDH protein. Only L*RDH*SN/Hep G2 cells expressed a detectable amount of protein by Western blot analysis, and the protein was detected in the microsomal fraction.

To further ensure the success of 9cRDH transduction, an enzyme activity test was performed using normal phase HPLC. Whole cell homogenates (100 μg) from L*X*SN/Hep G2 or L*RDH*SN/Hep G2 were incubated with 10 mM 9c-ROL and 2 mM NAD^+ for 60 minutes at 37°C. Following the incubation, cells were extracted and subjected to HPLC analysis as described in the method section (page 8). L*RDH*SN/Hep G2 produced 7-fold more 9c-RAL compared to L*X*SN/Hep G2 (**Fig. 8**). 9c-RAL produced by L*X*SN/Hep G2 is attributed to endogenous gene.

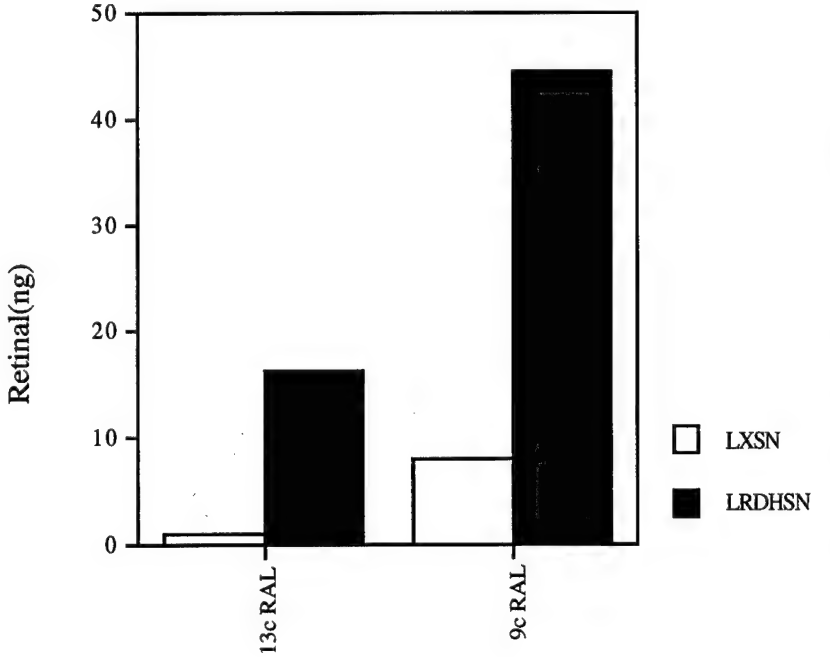

Figure 8. 9cRDH activity test by HPLC. Whole cell homogenates from L*X*SN/Hep G2 and L*RDH*SN/Hep G2 were incubated with 10 μM 9c-ROL for 60 minutes. 13c-RAL was detected due to both the isomerization from 9c-RAL and the production from its alcohol.

Enzyme kinetic studies

To further characterize the enzyme, kinetics studies were performed with microsomal fractions from L*RDH*SN/Hep G2. Microsomes (30 μg) of L*RDH*SN/Hep G2 were incubated with 0, 1, 2.5, 5, 10 μM 9c-ROL for 20 min. For each concentration, reactions were duplicated, and the same experiment was repeated 3 times. With an increase in substrate concentration, 9c-RAL production was increased in a linear fashion with up to 5 μM 9c-ROL, but enzyme seems to start getting saturated beyond it (**Fig. 9**). However, complete saturation of the enzyme was not observed, which may be attributed to the insolubility of 9c-ROL in Hepes buffer. Double reciprocal plot of substrate concentration and velocity was generated to calculate the apparent K_m, which varied among three experiments between 5 and 10 μM.

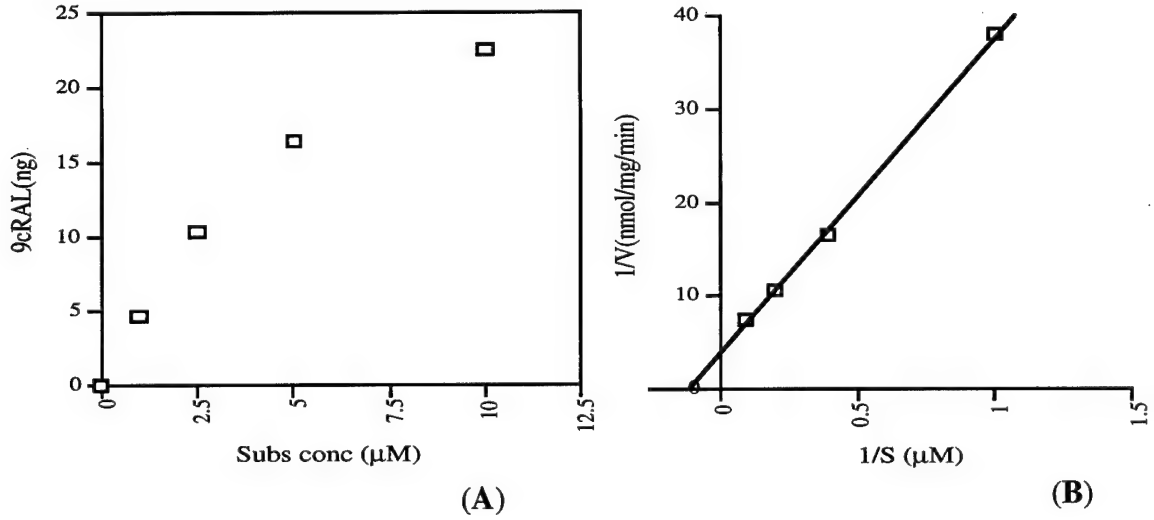

Figure 9. Enzyme kinetic studies. Microsomes of L*RDH*SN/Hep G2 cells were incubated with 9c-ROL at various concentrations for 20 minutes. Production of 9c-RAL was plotted against substrate concentration (**A**), and its double reciprocal plot (**B**) was generated to calculate the apparent K_m value.

Enzyme activity of breast cells

The immunoblotting assay used to show the existence of 9cRDH in breast cells has been a challenge due to low levels of the 9cRDH protein. Nonetheless, the enzyme activity test was performed using HPLC since even very low amounts of 9c-RAL (~1 ng) can be detected. Whole cell homogenates were incubated with 10 μM 9c-ROL and 2 mM NAD^+ for 60 min, and retinoids were extracted and analyzed as described in the method section (page 8). Since both 13c- and 9c-ROL can serve as the substrate for 9cRDH (35), and they were both detected at the end of reaction period, likely from photoisomerization, the sum of 9c- and 13c-retinal produced during this incubation was calculated and used to represent the total enzyme activity for each cell line. To compare the relative activity, these values were adjusted for that of AG11132. The summary of the data is presented in **Fig. 3** in which a comparison was made with mRNA expression studies. Protein levels parallel the mRNA expression. Moreover, our data suggests that the 9cRDH enzyme may play a major role in the oxidation of 9c-ROL *in vivo*.

Discussion

In contrast to our initial observations, semiquantitative, multiplex PCR expression analysis showed no evidence of diminished expression of 9cRDH mRNA in breast cancer

cells. Since the expression study was done using PCR, we can not rule out the possibility that the expressed mRNA may not be translated into proteins or that there are point mutations or other mutations outside the tested region of 9cRDH primers (cDNA sequence 397-870). Thus, we developed a functional test based on the activity assays developed using Hep G2 cells overexpressing 9cRDH. The activity test results paralleled the expression study data, suggesting that the expressed mRNAs are likely translated into functional proteins.

Recent studies from a number of laboratories provide evidence for an increasing number of enzymes that can metabolize either the *trans* and/or *cis* forms of retinols (21-23, 26, 30-35). These proteins and their genes belong to the growing family of short chain alcohol dehydrogenases (25). Since we have not purified the 9cRDH from breast cells to test its activity, a possible role for other enzymes with 9c-ROL oxidative activity can not be ruled out. However, we currently assume that 9cRDH is playing a major role in 9c-ROL metabolism in human mammary tissue, since the cDNA of 9cRDH was originally cloned from a normalized human mammary library and has been reported to be expressed at a high level in the normal mammary gland while other *cis*-retinol dehydrogenases were reported to be more abundant in the liver and kidney (26, 34).

A discrepancy in the mRNA expression of 9cRDH was observed between the breast tissue and breast cancer cells or normal HMECs. Mertz et al. (35) showed that tissue from a normal mammary gland expressed the mRNA at a very high level compared to its cancerous counterpart, which apparently showed a lack of expression as observed by Northern blot. However, we didn't see this pattern in cultured cells (**Figs. 2 and 3**). This difference can be explained as follows. The expression patterns observed in breast tissues might be confined to the particular samples in the Mertz study. Without further studies with well defined tissue samples, it is difficult to conclude whether there is a decreased expression in cancerous tissue. On the other hand, the expression level may have altered in a tissue culture system due to the separation from the natural environment, as observed in expression of cellular retinoic acid binding protein II (CRABPII) in keratinocytes that needed dermal-epidermal interaction (50). Thus, in our mRNA expression study, the cell culture system might not reflect what is happening *in vivo*.

Studies have shown that metabolism of all-*trans*-retinol varies between normal and cancerous cells of breast in that normal cells can esterify most of the retinols that are taken up to retinyl esters while cancer cells retain them as retinol (51). Thus, the lower expression of 9cRDH seen in normal HMECs in culture might be due to lower substrate availability . The source of 9c-ROL *in vivo* is still controversial. Nonetheless, the conversion of at-ROL to 9c-ROL by photoisomerization occurs *in vitro* and this conversion has been also reported *in vivo* (36). If this conversion is the source for 9cROL, its availability might be limited in normal cells, where most of all-*trans* retinol is converted to retinyl esters.

Moreover, tissue culture conditions may not be optimal for the expression of the 9cRDH. In our hands, mRNA transcripts of 9cRDH couldn't be detected from cells by Northern blot analysis whereas Mertz et al. reported very strong signal in a breast tissue using the same technique. It is highly likely that the expression of 9cRDH is tightly regulated since it could be the rate limiting step in 9c-RA production, a natural ligand for both RARs and RXRs. To date, we lack information on how 9cRDH is transcriptionally regulated. Breast cancer cells were grown in 5% serum supplemented media, which contains some retinoids, both retinols and retinoic acids. If a feedback regulation through the end product occurs for 9cRDH, it is possible that exogenous retinoic acids might decrease its expression in a tissue culture system. Thus, serum free tissue culture system may be required to assess this issue.

In the future, we plan to carry out the following experiments to determine the source of the discrepancy between tissue and cell culture system:
1. Analyze the expression of 9cRDH in a panel of normal and cancerous breast tissue from biopsy samples using semiquantitative, multiplex RT-PCR.
2. Examine breast cancer cells and normal HMECs for 9cRDH expression under various culture environments (± various retinoids) using semiquantitative, multiplex RT-PCR.
3. Measure retinoids in cultured cells grown in media with diverse retinoids and correlated them with expression of 9cRDH in the above experiment 2.

Recently, other investigators have shown that retinoid metabolizing enzymes may use various steroids as their substrates (26, 51, 52). Since then, the identity of h9cRDH has been debated: is 9cRDH really a 9c-ROL oxidizing enzyme or another steroid metabolizing enzyme which can use retinol *in vitro* ? Or is it a bifunctional enzyme capable of using both substrates depending on the environment? In either case, this could have a profound importance in mammary gland development and/or homeostasis. Our collaborator, Dr. Blaner, has been testing various steroids as possible substrates and so far none of them has been found to be a better substrate than 9c-ROL for 9cRDH, using thin layer chromatography.

Since 9c-retinoic acid has been identified as a biological ligand for RXR (27, 28), interest has been increased to identify a pathway for 9c-RA synthesis *in vivo*. The simplest pathway, isomerization of at-RA to 9c-RA has been suggested based on the observation in cells and tissue homogenates (27-29). Even though an isomerization pathway is possible, a non-enzymatic one seems less likely to be the main mechanism for *in vivo* synthesis of 9c-RA. An alternative pathway has been put forward following the identification of various *cis*-retinol dehydrogenases. If indeed 9c-RA is the biological ligand for RXR, a very precise regulation of the levels of enzymes involved in oxidation of the precursor seems to be necessary. RXRs are required for the transcriptional regulation of numerous genes, not only through homodimerization but also through heterodimerization with different nuclear receptors, such as RAR, thyroid receptor (TR), vitamin D receptor (VDR) and peroxisomal proliferator activated receptor (PPAR). Thus, 9c-RA via non-enzymatic isomerization might make it difficult to control many of the downstream genes whose expression is dependent on the signaling by these receptors.

Based on these collective studies, we suggest that 9cRDH might play a major role in 9c-RA synthesis *in vivo*. To explore the importance of the gene in human mammary tissue, following experiments are planned and/or in progress.
1. We are currently developing MCF-7 cells transduced with 9cRDH (L*RDH*SN/MCF-7) and with LXSN (LXSN/MCF-7) using a retroviral vector system.
2. We are planning to follow the retinoid metabolism in L*RDH*SN/MCF-7 cells under different culture conditions (various retinoids) and the comparison will be made to LXSN/MCF-7 and parental MCF-7.
3. We also plan to follow retinoid metabolism in HMECs to identify differences, if any, between normal and cancer cells.

Conclusions

I have shown that 9cRDH is involved in 9c-ROL oxidation to 9c-RAL. In addition, I've found that the mRNA expression of this enzyme varied among breast cells, MCF-7 being the lowest and T47D and Hs578T being the highest. Two normal HMECs exhibited lower to intermediate level of expression of 9cRDH.

A model system to study the biochemical characteristics of 9cRDH has been developed with Hep G2 cells using a retrovirus system. Kinetic studies showed that the

9cRDH enzyme follows Michaelis-Menten kinetics, and the calculated apparent K_m was 5-10 μM.

Summary of statement of work

1. RNA in situ

Several probes were made from different regions of the 9cRDH cDNA gene for an RNA *in situ* hybridization study to localize 9cRDH in breast tissue using Digoxigenin (Dig) -labeling system (Boehringer-Mannheim). Initially, the full length cDNA was used to make riboprobes, followed by subsequent alkaline hydrolysis to give an optimal length of between 200-600 bps. In addition, to ensure that the sense- and antisense-probes are made from the same region of the gene, subcloning was performed to create vectors containing ~360bp of the 5' fragment of 9cRDH. Double digestions with EcoRI and XhoI eliminated the 3' fragment of ~870 bp from the 9cRDH gene in pcDNA3 where it is originally cloned. Following the digestion, a fill-in reaction was performed using Klenow enzyme (Boehringer-Mannheim), and the linearized vector containing 360bp 9cRDH was religated using T_4 ligase at 14°C overnight. The resulting subclone was verified for its sequence using the Sequenase kit® (Amersham). *In situ* hybridization studies have been delayed in order to stably transduced Hep G2 cell to use as a model system for enzyme characterization.

2. RT-PCR

RT-PCR was performed on mRNA from cultured normal and tumor cells with various combinations of 9cRDH primers. In order to quantitate the mRNA expression level more accurately, a semiquantitative RT-PCR assay was developed. A panel of breast cancer cells and two normal HMECs have been tested with a combination of RDH primers and HPRT primers (control). This first set of experiments has been completed and currently, a study with a second set (different RDH primers) of primers is in progress to confirmed the previous results. 9cRDH mRNA induction by retinoic acid has not been tested due to a delay in the development of semiquantitative PCR. We are planning to expand these experiments to include more diverse retinoids such as 9c-ROL and 9c-RA.

3. Metabolism and growth studies with breast cells

Growth curve studies have not been initiated as I wish to carefully determine the cell line to be analyzed. We will choose two breast cancer cell lines, one that exhibits low levels and one that exhibits high levels of 9cRDH by RT-PCR. The metabolism and growth studies will be carried out as soon as the semiquantitative RT-PCR studies with the second set of primer combinations is completed.

For the metabolism studies in breast cancer cells, we have revised our plan and developed a stable transduction model instead of transient transfection, because stable transduction will generate homogeneous populations of clonal cell lines. We feel this will increase the reproducibility in the metabolism studies since the gene transfection efficiency would not be a problem as in case of transient transfection. We are currently developing 9cRDH transduced MCF-7 cells because these cells had the lowest expression and activity levels based on RT-PCR and activity test results.

4. Immunoblotting assays and biochemical characterization of 9cRDH

Immunoblotting assays and kinetic studies of 9cRDH have been included. This represents a modification of the original proposal. Protein levels should be the "gold standard", since mRNA expression does not guarantee translation of 9cRDH. We have tested a polyclonal anti-peptide antibody on a diverse group of breast cancer cells and HMECs. However due to low endogenous protein levels of 9cRDH, immunoblotting has

been a challenge unlike with Hep G2 cells transduced with 9cRDH, where the protein detection was fairly easy. We are currently developing another antibody which will be raised against a recombinant protein.

Due to low expression of 9cRDH in the breast cells, the L*RDH*SN/Hep G2 cell line was created for enzyme kinetic studies using the retroviral vector system. This cell line has also served as a positive control for immunoblotting assays.

References

1. Marth, C., Mayer, I., and Gunter, D. (1984) *Biochem. Pharmacol.* **33,** 2217-2221
2. McCormick, D., Burns, F., and Albert, R. (1981) *J. Natl. Cancer. Inst.* **66,** 559-564
3. McCormick, D., and Moon, R. (1982) *Cancer Res.* **42,** 2639-2643
4. McCormick, D., Sowell, Z., Thompson, C., and Moon, R. (1983) *Cancer* **51,** 594-599
5. Mehta, R., Hultin, T., and Moon, R. (1988) *Biochem. J.* **256,** 579-584
6. Miorana, A., and Gullino, P. (1980) *J. Natl. Cancer. Inst.* **64,** 655-663
7. Moon, R., Grubbs, C., and Sporn, M. (1976) *Cancer Res.* **36,** 2626-2630
8. Moon, R. et al. (1979) *Cancer Res.* **39,** 1339-1346
9. Moon, R., Pritchard, J., Mehta, R., Nomides, C., Thomas, C., and Dinger, N. (1989) *Carcinogenesis* **10,** 1645-1649
10. Moon, R., Mehta, R., and Detrisac, C. (1992) *Cancer Detect. Prev.* **16,** 73-7911.
11. Moon, R., Mehta, R., and Rao, K. (1994) in *The retinoids: Biology, chemistry and medicine* (Sporn, M., Roberts, A., and Goodman, D., eds), 2nd Ed., pp. 573-595, Raven Press, New York
12. Sukumar, S., McKenzie, K., and Chen, Y. (1995) *Mutat. Res.* **333,** 37-44
13. van der Burg, B., van der Leede, B. M., Kwakkenbos-Isbrucker, L., Salverda, S., Laat, S. W., and van der Saag, P. T. (1993) *Mole. Cell. Endocrinol.***91,** 149-157
14. Zhao, Z., Zhang, Z.-P., Soprano, D. R., and Soprano, K. J. (1995) *Exp. Cell Res.* **219,** 555-561
15. Gottardis, M. M., Lamph, W. W., Shalinsky, D. R., Wellstein, A., and Heyman, R. A. (1996) *Breast Cancer Res. Treat.* **38,** 85-96
16. Blaner, W., and Olson, J. (1994) in *The retinoids: Biology, chemistry and medicine* (Sporn, M., Roberts, A., and Goodman, D., eds), 2nd Ed., pp. 229-255, Raven Press, New York
17. Zgombic-Knight, M., Foglio, M. H., and Duester, G. (1995) *J. Biol. Chem.*. **270,** 4305-4311
18. Duester, G. (1996) *Biochemistry* **35,** 12221-12227
19. Ang, H., Deltour, L., Zgombic-Knight, M., Wagner, M., and Duester, G. (1996) *Alcohol. Clin. Exp. Res.* **20,** 1050-64
20. Ang, H., Deltour, L., Hayamizu, T., Zgombic-Knight, M., and Duester, G. (1996) *J. Biol. Chem.* **271,** 9526-9534
21. Chai, X., Zhai, Y., Popescu, G., and Napoli, J. L. (1995) *J. Biol. Chem.* **47,** 28408-28412
22. Chai, X., Boerman, M., Zhai, Y., and Napoli, J. (1995) *J. Biol. Chem.* **270,** 3900-3904
23. Chai, X., Zhai, Y., and Napoli, J. (1996) *Gene* **169,** 219-222
24. Tsigelny, I., and Baker, M. E. (1996) *Biochem. Biophys. Res. Commun.* **226,** 118-127
25. Jornvall, H., Persson, B., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffery, J., and Ghosh, D. (1995) *Biochem.* **34,** 6003-6013
26. Su, J., Chai, X., Kahn, B., and Napoli, J. L. (1998) *J. Biol. Chem.* **273,** 17910-17916
27. Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M., and Thaller, C. (1992) *Cell* **68,** 397-406
28. Levin, A. et al. (1992) *Nature* **355,** 359-361
29. Urbach, J., and Rando, R. R. (1994) *Biochem. J.* **299,** 459-465
30. Driessen, C. A. G. G., Janssen, B. P. M., Winkens, H. J., van Vugt, A. H. M., de Leeuw, T. L. M., and Janssen, J. J. M. (1995) *Invest. Ophthalmol. Vis. Sci* . **36,** 1988-1996
31. Driessen, C., Winkens, H., Kuhlmann, E., Janssen, A., van Vugt, A., Deutman, A., and Janssen, J. (1998) *FEBS Letters* **428,** 135-140

32. Romert, A., Tuvendal, P., Simon, A., Dencker, L., and Ulf, E. (1998) *Proc. Natl. Acad. Sci, USA* **95,** 4404-4409
33. Simon, A., Hellman, U., Wernstedt, C., and Eriksson, U. (1995) *J. Biol. Chem.* **270,** 1107-1112
34. Chai, X., Zhai, Y., and Napoli, J. (1997) *J. Biol. Chem.* **272,** 33125-33131
35. Mertz, J. R., Shang, E., Piantedosi, R., Wei, S., Wolgemuth, D. J., and Blaner, W. S. (1997) *J. Biol. Chem.* **272,** 11744-11749
36. Creech-Kraft, J., Kimelman, D., and Juchau, M. R. (1995) *Drug Metab. Dispos.* **23,** 72-82
37. Levin, G., and Mokady, S. (1994) *Internat. J. Vit. Nutr. Res.* **64,** 165-169
38. You, C.-S., Parker, R. S., Goodman, K. J., Swanson, J. E., and Corso, T. N. (1996) *Am. J. Clin. Nutr.* **64,** 177-183
39. Labrecque, J., Dumas, F., Lacroix, A., and Bhat, P. V. (1995) *Biochem. J.* **305,** 681-684
40. Brown, P., Blum, W., and Stern, M. (1959) *Nature* **184,** 1377-1379
41. Matssui, M. et al. (1958) *J. Vitaminol.* **4,** 178-189
42. Aden, D., Fogel, A., Plotkin, S., Damjanov, I., and Knowles, B. (1979) *Nature* **282,** 615-616
43. Knowles, B., Howe, C., and Aden, D. (1980) *Science* **209,** 497-499
44. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) in *Molecular Cloning: a Laboratory Manual* (Nolan, C., ed) Vol. 1, Second Ed., pp. 7.19-7.22, 3 vols., Cold Spring Harbor laboratory Press, New York
45. Henegariu, O., Heerema, N., Dlouhy, S., Vance, G., and Vogt, P. (1997) *BioTechniques* **23,** 504-511
46. Jolly, D., Okayama, H., Berg, P., Esty, A., Filpula, D., Bohlen, P., Johnson, G., Shively, J., Hunkapillar, T., and Friedmann, T. (1983) *Proc. Natl. Acad. Sci.* **80,** 477-481
47. Miller, A., Miller, D., Garcia, J., and Lynch, C. (1993) *Methods Enzymol.* **217,** 581-599
48. Napoli, J. (1986) *Methods Enzymol.* **123,** 112-124
49. Fleischer, S., and Kervina, M. (1974) *Methods Enzymol.* **31,** 6-41
50. Elder, J. T., Astrom, A., Pettersson, U., Tavakkol, A., Griffiths, C. E., Krust, A., Kastner, P., Chambon, P., and Voorhees, J. J. (1992) *J. Invest. Dermatol.* **98,** 673-679
51. Chen, A. C., Guo, X., Derguini, F., and Gudas, L. J. (1997) *Cancer Res.* **57,** 4642-4651
52. Biswas, M., and Russell, D. (1997) *J. Biol. Chem.* **272**(25), 15959-15966

Abstract: Retinoids '97, Nice, France, September 28 - October 1, 1997

Characterization of 9-cis-retinol dehydrogenase

M. Gamble*, J. Paik§, J. R. Mertz*, E. Shang*, K. Swisshelm§, D. J. Wogelmuth*, W. S. Blaner*. Columbia University, New York, NY*, and University of Washington§

We have identified and characterized a 1.4 kb cDNA clone which encodes a protein that catalyzes oxidation of 9-*cis*-retinol (9cROH) to 9-*cis*-retinaldehyde, referred to as human 9-*cis*-retinol dehydrogenase (h9cRDH). This clone was obtained from a normalized human mammary tissue cDNA library. Messenger RNA fro h9cRDH has been shown by Northern blot to be present most prominently in mammary gland, testis and kidney, with smaller amounts in liver, heart and adrenals. h9cRDH cDNA was cloned into the eukaryotic expression vector, pcDNA3, and used to transiently transfect CHO cells. Cell homogenates from transfected CHO cells served as a source of h9cRDH activity for further enzyme characterizations. These characterizations have revealed that h9cRDH catalyzed the oxidation of 9cROH at a maximal rate of 84 pmol/min/mg protein, and that h9cRDH does not show appreciable activity towards all-*trans*-retinol. This protein shares 50% identity at the amino acid level with rat all-*trans*-retinol dehydrogenases, type I, II, and III, 89% identity at the amino acid level to bovine 11-*cis*-retinol dehydrogenase (11cRDH), and 97% identity to the recently reported human 11cRDH. Of the eight amino acids which differ between the h9cRDH and the h11cRDH, seven occur in the 2nd exon (the first coding exon), and seven are non-conservative substitutions. Since preliminary studies indicate that the h9cRDH can catalyze oxidation of 11-*cis*-retinol at a rate which is approximately equivalent to that of 9cROH and considering the high degree of identity between h9cRDH and h11cRDH, this raises a question as to whether 9cRDH and h11cRDH are distinct enzymes. The literature indicates that bovine 11cRDH is present only in the eye, and that it cannot catalyze the oxidation of 9cROH. This would suggest h9cRDH and 11cRDH are indeed distinct enzymes. We are presently carrying out experiments designed to distinguish whether h9cRDH and 11cRDH are distinct enzymes.

Abstract: 1998 FASEB Summer Research Conferences, Snowmass Village, June 13-18, 1998

Expression and activity of a 9-cis retinol dehydrogenase in normal human mammary epithelial cells and breast cancer cells

Jisun Paik*, William S. Blaner§, and Karen Swisshelm*. University of Washington, Seattle WA* and Columbia University, New York, NY§

Retinoids may negatively modulate the growth, initiation and progression of breast cancer in cell culture and animal models. On mechanism by which retinoic acids affect cell growth is through binding and activation of the nuclear receptors, the RARs and RXRs. Little is known about the synthesis of retinoic acids in tissues such as the mammary gland. Until recently it was thought that 9-*cis* retinoic acid, the favored ligand for RXRs, was formed by the isomerization of all-*trans* retinoic acid. Several investigators have recently cloned the genes for retinol dehydrogenases, including 9-*cis* retinol dehydrogenase (9cRDH). Blaner and colleagues demonstrated that the gene product catalyzes 9-*cis* retinol oxidation to 9-*cis* retinaldehyde. Since the cDNA was isolated from a normalized human mammary library, we studied its expression in two normal human mammary epithelial cell strains (HMECs) and a panel of breast cancer cell lines. mRNA levels were determined by a semi-quantitative, multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) strategy. 9cRDH was similarly expressed among the cells tested, with exception of the breast cancer cell lines, Hs578T and T47D exhibiting higher expression than the normal strains and MCF-7 cells showing the lowest levels among all cells examined. Protein levels examined by immunoblotting, using a polyclonal antipeptide antibody, paralleled expression levels observed for mRNA. In order to test and compare activity of 9cRDH in cell lines, we developed a Hep G2 cell line that expresses high levels of 9cRDH protein. The 9cRDH cDNA was transduced into the cells using the LXSN retroviral system. Individual 9cRDH activity levels in normal and tumor cells, as measured by normal phase HPLC, again paralleled expression levels observed by RT-PCR and immunoblotting. Activity of 9cRDH tested in microsomes of LRDHSN/Hep G2 cells was time- and protein concentration-dependent and followed Michaelis-Menten kinetics.